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Article Addendum

Two different signaling pathways for thaxtomin A-induced cell death in Arabidopsis and tobacco BY2

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Abbreviations: $[Ca^{2+}]_{cyt}$, cytosolic calcium concentration; FDA, fluorescein-diacetate; HR, hypersensitive response; MAPK, mitogen-activated protein kinases; PCD, programmed cell death; PM, plasma membrane; ROS, reactive oxygen species; TXT, thaxtomin A

Key words: *Arabidopsis thaliana*, calcium, cell death, *Nicotiana tabacum* BY2, plant pathogen, thaxtomin A

Thaxtomin A (TXT) is a phytotoxin produced by all plant-pathogenic *Streptomyces scabies* involved in the potato scab disease. Their pathogenicity was previously correlated with the production of TXT. Calcium is known to be an essential second messenger associated with pathogen-induced plant responses and cell death. We have effectively shown that in *Arabidopsis thaliana* cell suspensions, TXT induces an early short lived Ca^{2+} influx which is involved in the cell death process and other TXT-induced responses. We extended our study to *Nicotiana tabacum* BY2 by monitoring cell death and changes in cytosolic calcium concentration on cells expressing the apoaequorine Ca^{2+} reporter protein to compare the responses to TXT of the two model plants, tobacco and *A. thaliana*. Our investigations show that cell death in BY2 appeared to be dose dependent with a lag of sensitivity comparing to *A. thaliana*. Moreover, pathway leading to cell death in BY2 does not involve calcium signaling. Our results suggest that different pathways are engaged in *A. thaliana* and *N. tabacum* BY2 to achieve the same response to TXT.

Plants are constantly exposed to pathogens and have evolved a diversity of responses in order to withstand these attacks. Recognition and perception of a pathogen or their derived-elicitors by plant cells lead to modulation of the defence-signaling pathways including: production of reactive oxygen species (ROS), modulation of ion fluxes, increase of cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$), activation of mitogen-activated protein kinases (MAPK) and expression of defence-related genes.[1,2] Additionally, plants often induce a hypersensitive response (HR) characterized by a localized cell death often associated with disease resistance.[3] This form of programmed cell death (PCD)[4] requires gene expression and metabolic activities[3] that displays apoptosis-like features. Thaxtomin A (TXT) is a nitrated dipeptide phytotoxin produced by all plant-pathogenic of the Streptomyces species.[5,6] TXT inhibits the cellulose synthesis, suggesting that cell wall is one of the main target of TXT.[7,8] In the roots of different species, TXT stimulates H^+ efflux across plasma membrane (PM) and a short lived Ca^{2+} influx, inhibited by La^{3+}, a PM Ca^{2+} channel inhibitor.[9] TXT also induces cell death depending on active gene transcription and de novo protein synthesis, which are PCD hallmarks.[10] We recently reported new insights regarding the effect of TXT[11] by using *A. thaliana* suspension cells, a convenient material for studying early physiological events induced by pathogens.[10,12-15] We showed that the transient increase in $[Ca^{2+}]_{cyt}$ was a key step of a further signaling pathway leading to the death of the cells in response to a TXT accordingly to previous studies which showed that Ca^{2+} activity changes are involved in PCD in plant, as in animal.[16,17] Fast rises in $[Ca^{2+}]_{cyt}$ are effectively also frequently described as one of the earliest responses to various microbial phytotoxins and elicitors[18,19] and numerous studies led to the conclusion that activation of defence responses depends on Ca^{2+} influxes from the apoplast into the cytosol of plant cells.[16] Notably, elicitor-induced uptake of Ca^{2+} from the extracellular medium was shown to be required for the controlled generation of H_2O_2,[20-22] the activation of MAPK pathways,[2,23] the activation of defence related genes[24] and production of phytoalexin.[16] In the present work, we extended our study by comparing the TXT-induced responses in *A. thaliana* and *N. tabacum* BY2 suspension cells. The cell death detection experiments and Ca^{2+} assay we performed demonstrate that the signal transduction pathways leading to the cell death in response to TXT are different in these two plant models.

Arabidopsis thaliana L. suspension cells were grown in Gamborg medium (pH 5.8) and *Nicotiana tabacum* BY2 suspension cells were grown in Murashige and Skoog medium (pH 5.8). All experiments were performed using log-phase cells (4 days after sub-culture for Arabidopsis and 6 days for BY2). Cell death was quantified using

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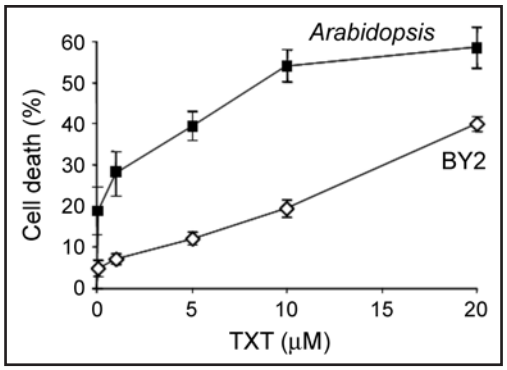

Figure 1. Effect of increasing concentrations of TXT, on FDA estimated cell death increase after 6 h of treatment for *Arabidopsis thaliana* (■) and *Nicotiana tabacum* BY2 (◇) cells. The data correspond to the means of 3 replicates during one experiment and error bars correspond to standard errors. Data are representative of at least 3 independent experiments.

the fluorescein diacetate (FDA) spectrofluorimetric method.[11] Cell death appeared to be dose dependent for both suspension cells (Fig. 1) with a lag of sensitivity in BY2. In *A. thaliana* cells, the cell death plateau, around 50%, was reached within 6 hours for 10 µM of TXT, while in BY2 only 20% of cells were dead (Fig. 1). In BY2 cells, only 40% of cell death was reached within 6 hours for 20 µM of TXT (Fig. 1). The further comparisons were done with TXT concentrations inducing about the same extent of cell deaths, 10 µM and 20 µM of TXT for Arabidopsis and BY2, respectively. In *A. thaliana* cells, 10 µM TXT induces a rapid influx of Ca^{2+} through the PM inhibited by La^{3+}, Gd^{3+} or BAPTA.[11] In BY2 cells, also expressing aequorin in their cytosol, no significant increase in $[Ca^{2+}]_{cyt}$ were recorded upon addition of 20 µM TXT (Fig. 2A). In *A. thaliana* cells, the influx of Ca^{2+} is an upstream event in the signaling pathway leading to TXT-induced cell death.[11] We thus compared the effect of a 6 hours treatment with TXT on both cell lines in presence or absence of La^{3+}, a PM Ca^{2+} channel inhibitor. As expected, La^{3+} pretreatment of BY2 cells failed to decrease the TXT-induced cell death (Fig. 2B), contrarily to what observed for *A. thaliana*. These data suggest that Ca^{2+} is no involved in the signaling pathway leading to cell death in *N. tabacum* BY2 in response to TXT. However, in *A. thaliana* a Ca^{2+} independent pathway could although exists. The analysis of mRNA levels after treatment of the cells with 10 µM TXT by RT-PCR shows an increase for *PAL1*[11] and *AtHSR4* genes (Fig. 3), but only *PAL1* induction seemed to be Ca^{2+} dependent since addition of La^{3+} decreased the accumulation of the transcripts of *PAL1,* coding for a key enzyme of the phenylpropanoid pathway, but not the accumulation of the transcripts of *Hsr4*, which encodes an AAA type ATPase, reported to be rapidly induced in tobacco after the onset of HR by TMV and incompatible *Pseudomonas syringae*.[25] These experiments suggest that different signaling pathways could be induce in response to TXT in the same cells and highlight the different behaviors which could be observed among plant models even to achieve the same goal, cell death and defense response.

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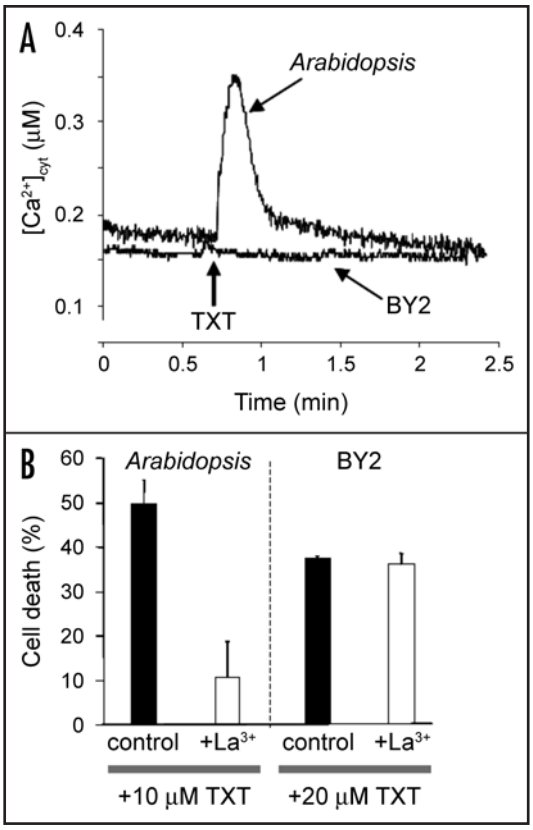

Figure 2. (A and B) Role of Ca^{2+} in TXT-induced responses in *Arabidopsis thaliana* and *Nicotiana tabacum* BY2 suspension cells. (A) Changes in $[Ca^{2+}]_{cyt}$ were measured by using cell suspensions derived from Arabidopsis and BY2 expressed the apoaequorin gene, upon 10 µM and 20 µM TXT addition, respectively. Data are representative of 5 independent experiments. (B) Effect of a pretreatment with La^{3+} (500 µM), a plasma membrane Ca^{2+} channel inhibitor, on TXT-induced cell death after 6 h. The data correspond to the means of 3 replicates during one experiment and error bars correspond to standard errors. Data are representative of at least 3 independent experiments.

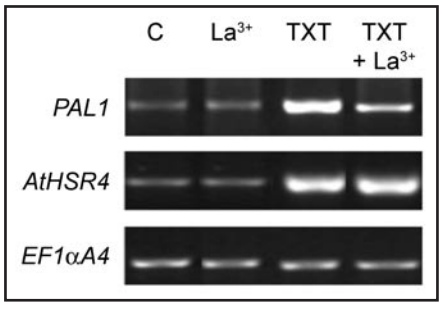

Figure 3. Involvement of calcium influx on defence-related gene expression in *Arabidopsis thaliana* suspension cells after treatment with 10 µM TXT during 4 h. Effect of La^{3+} on expression of *PAL1*, a defence-related genes, and *Athsr4*, an early marker of the hypersensitive response, in response to 10 µM TXT (C: control without TXT). Cells were pre-incubated with La^{3+} (500 µM) for 15 min before TXT addition. RT-PCR were performed with total RNA extracted from *A. thaliana* cells. *EF1αA4* gene was used as a control to analyze quality of RNA and to normalize the different samples for differences in the amount of RNA.

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